Exhibit 2

quency, these are the same microwaves used for applications such as wireless access to the Internet. Different materials on a person's body reflect them to varying degrees, enabling a computer to generate a three-dimensional image showing the outlines of concealed objects. Because the waves are nonionizing, "they do not pose any health risks," Sheen says. The scan currently takes up to 10 seconds, during which the person must stand relatively still. Generating the image takes up to another 30 seconds. Sheen says his group is working to speed up the system.

A similar technology described by Robert Barat, a chemical engineer at the New Jersey Institute of Technology in Newark, uses waves of a shorter wavelength. Submillimeter (or terahertz) waves, familiar to radio astronomers, generate a spectrum when they interact with a material. They can also be transmitted farther than millimeter waves can. By harnessing those properties, Barat's group hopes to design scanners that would be capable of detecting weapons and bombs carried by a terrorist more than 5 meters away. The method "has the potential of not only showing the presence of a hidden substance but also of identifying the substance based on a transmission or reflection spectrum," says Barat, who has yet to build a prototype. Jehuda Yinon, an expert on explosives detection at the Weizmann Institute of Science in Rehovot, Israel, says the technology could be an invaluable tool for identifying suicide bombers in public places.

Other talks spotlighted new biosensors for detecting chemical and biological agents. Their common goal is to sniff out smaller and smaller doses of toxins in the environment with greater speed and accuracy. For exa

a molecular cylinder made up of

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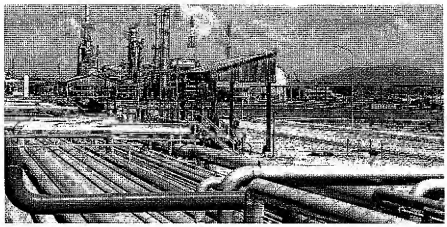

Cheaper gas? Converting waste into H_2 could lower refining costs and spur a hydrogen economy.

New Routes Toward Practical Hydrogen?

Hydrogen makes a tantalizing fuel. Water is its only byproduct when burned or run through a fuel cell to make electricity. It's also the most abundant element in the universe. But the downside is that earthly hydrogen is almost always bound to other elements, and liberating it requires much more energy than it releases as a fuel. At the meeting, two separate teams reported novel approaches to extracting hydrogen from waste products that could bring a sustainable hydrogen economy a step closer.

In the first, researchers from Pennsylvania and Georgia reported on a new catalyst that converts hydrogen sulfide (H_2S)—an abundant contaminant in natural gas wells—to hydrogen gas (H_2). In the other, researchers from Indiana revealed a new process for recovering H_2 from silicon-based compounds, which could open the door to new ways of generating and storing hydrogen.

Outsiders say it's too early to tell whether these approaches make economic sense. But they are "promising avenues," says Joseph Sadighi, a catalyst expert at the Massachusetts Institute of Technology in Cambridge.

Raiding industrial waste for useful chemicals is nothing new. H_2S is routinely converted to sulfur dioxide (SO_2) as part of a process to generate sulfuric acid, a widely used compound in the chemical industry. But although that reaction turns the sulfur in H_2S into a valuable commodity, it misses an opportunity to do the same for hydrogen by instead converting it to water.

Using vanadium-based catalysts to convert H_2S into SO_2 can generate H_2 instead of water, report Israel Wachs of Lehigh University in Bethlehem, Pennsylvania, and Andrew Gibson, who heads Gibson Technologies in Atlanta, Georgia. The conversion, Gibson explained, takes place in two steps. First, carbon monoxide (CO) reacts with H_2S using a long-known reaction to generate H_2 and another compound called carbonyl sulfide (COS), a toxic byproduct. The COS is then fed to another chamber, where it reacts with oxygen over a vanadium oxide catalyst to form SO_2 and CO. The CO is then fed back into the first reaction to generate more H_2.

Unlike the current technology used to convert H_2S to H_2, which extracts the CO needed for the hydrogen-generating reaction from expensive natural gas, the new approach continually generates CO by breaking down the toxic COS. Gibson notes that the process not only might fuel a future hydrogen economy but also could reduce the cost of refining gasoline by supplying H_2 needed to strip crude oil of sulfur.

Purdue University chemist Mahdi Abu-Omar and colleagues offered a very different scheme for generating hydrogen. They discovered it while looking for novel catalysts to convert organic silicon-based liquids called organosilanes into silanols, a more valuable class of compounds used in the chemical industry. The researchers were working with rhenium-based catalysts, which they added to organosilanes and water. They found that the rhenium catalysts not only readily converted their organosilanes into silanols but also generated large amounts of H_2. Organosilanes may make an attractive way to store hydrogen for later use in fuel cells, Omar notes, because both they and the silanol "wastes" are liquids and easy to transport.

Abu-Omar acknowledges that the compounds are somewhat costly to produce and are generated industrially in only small quantities. At the meeting, Sadighi noted that related catalysts might also react with another silicon-based liquid, called PMHS, which is produced in large quantities as a byproduct of the silicone business. Turning this or other more abundant organic compounds into hydrogen could make hydrogen an even more tantalizing fuel. –ROBERT F. SERVICE

sense extremely low concentrations of toxin. "What they've done is amplified the signal. It's really very clever," says James Robertson, a research biologist at the Federal Bureau of Investigation Laboratory in Quantico, Virginia.

—YUDHIJIT BHATTACHARJEE

ple, a new sensing technique described by Jeffrey Mason, a researcher at the Armed Forces Institute of Pathology in Washington, D.C., can detect as few as 500 molecules of cholera or botulinum in a sample. That's 1000 times more sensitive than existing techniques.

The heart of the sensing device is a liposome—a molecular cylinder made up of lipids—with a DNA molecule encapsulated inside and a receptor molecule on the outside that attaches specifically to the toxin. The toxin molecules are first captured on a plate using antibodies that bind to the toxin. When the liposomes are added to this mix, the receptor molecules linked to them attach to the toxin as well. At the end of the assay, everything else is washed away, leaving only the liposomes that have been chained to the toxin molecules.

The researchers then split the liposomes open with an enzyme to release the DNA molecules and tally them with a standard polymerase chain reaction (PCR)—in effect, using the DNA molecules as a proxy for the toxin. And because PCR can detect tiny amounts of DNA (by making many copies of DNA molecules present in a sample), the technique can

Liposome polymerase chain reaction assay for the sub-attomolar detection of cholera toxin and botulinum neurotoxin type A

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We describe an ultrasensitive immunoassay for detecting biotoxins that uses a liposome with encapsulated DNA reporters, and ganglioside receptors embedded in the bilayer, as the detection reagent. After immobilization of the target biotoxin by a capture antibody and co-binding of the detection reagent, the liposomes are ruptured to release the reporters, which are quantified by real-time polymerase chain reaction. The new assays for cholera and botulinum toxins are several orders of magnitude more sensitive than current detection methods. A single 96-well microtiter plate can analyze ~20 specimens, including calibration standards and controls, with all measurements conducted in triplicate. Using pre-coated and blocked microtiter plates, and pre-prepared liposome reagents, a liposome polymerase chain reaction assay can be carried out in about 6 h.

INTRODUCTION

The potential use of biological toxins as weapons of mass destruction has created an imperative to develop rapid, field-deployable and highly sensitive assays for the detection of these agents. In addition, assays for biological toxins have applications in such diverse fields as microbiology, clinical diagnostics, the evaluation of therapeutic agents, agriculture and environmental and food testing. Biological toxins typically exhibit extreme potency. For example, botulinum neurotoxin type A (BoNT/A), which is produced by the anaerobic bacterium *Clostridium botulinum*, is about 100 billion times more toxic than cyanide; it is the most lethal human toxin known, with an LD_{50} of approximately ng per kg^1 (ref. 1). Thus, assays for biological toxins must be not only highly specific, but also highly sensitive with the ability, in some applications, to detect toxins down to the level of a few hundred molecules. The only current assay technology capable of this level of sensitivity couples the protein detection specificity of antibody–protein binding with the powerful amplification capability of the polymerase chain reaction (PCR). Immuno-PCR, first introduced by Sano *et al.*[2], uses a reporter oligonucleotide that is either covalently[3] or non-covalently[3] attached to an antibody specific for the protein of interest. Although these methods allow for the highly specific and sensitive detection of protein targets, they have limitations that have prevented their widespread use. These shortcomings include poor sensitivity and reproducibility with complex environmental or biological specimens, the expense and short shelf life of the reagents, the complex synthesis necessary to fabricate the detection reagents and the high susceptibility of the assay to contamination with reporter oligonucleotide. Here we describe an ultrasensitive assay for the detection of biotoxins, which we call liposome polymerase chain reaction (LPCR)[4], that overcomes many of the limitations of conventional immuno-PCR. This assay uses a liposome with encapsulated DNA reporters, and ganglioside receptors embedded in the bilayer, as a detection reagent (Fig. 1). After immobilization of the target biotoxin in a microtiter plate well by a capture antibody and co-binding of the DNA-liposome detection reagent, the vesicles are ruptured to release the DNA reporters,

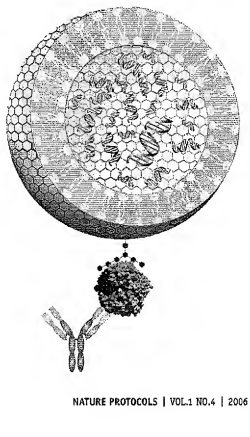

Figure 1 | Representation of a liposome detection reagent shown in cross section. The dsDNA reporters (green with red bars) are encapsulated inside the lipid bilayer of the liposome (yellow) into which monosialoganglioside G_{M1} receptors (blue) have been incorporated. The liposome is shown bound to a cholera toxin beta subunit (CTBS) pentamer, which is co-bound to a capture antibody.

which are quantified by real-time PCR. Encapsulation of reporters inside liposomes offers two advantages over current immuno-PCR methods. First, ~60 reporters can be encapsulated inside each liposome increasing the sensitivity of the assay. Second, any contaminating reporter DNA in the plate wells can be degraded by DNase digestion immediately prior to lysis of the liposomes, as the enzyme cannot pass through the liposomal bilayer. Limitations of LPCR relative to the simpler, but less sensitive, enzyme-linked immunosorbent assay (ELISA) include the need to prepare the DNA-liposome detection reagent and the requirement of performing real-time PCR. LPCR assays for cholera toxin beta subunit (CTBS) and BoNT/A yield detection thresholds below 1 fg ml^{-1}, which is 2–3 orders of magnitude more sensitive than current detection methods[5–7]. Other biotoxins that could potentially be detected using ganglioside receptors include tetanus, pertussis, shiga, ricin and heat-labile enterotoxin[8,9]. We are currently developing LPCR assays that employ antibodies as antigen receptors in place of gangliosides. These assays are being used to detect additional biotoxins as well as biomarkers for cancer, prion disease, dengue virus and human immunodeficiency virus.

MATERIALS

REAGENTS

- Monosialoganglioside G_{M1} from bovine brain (Sigma)
- Cholera toxin beta subunit (non-toxic subunit) from *Vibro cholerae* (CTBS, Sigma)
- Ficoll, 70 kDa (Sigma)
- DNase I from bovine pancreas (Type IV; Sigma)
- Exonuclease III from *Escherichia coli* (Sigma)
- Triton X-100, ultra grade (Sigma)
- Bovine serum albumin, RIA grade (BSA; Sigma)
- Sepharose CL-4B (Sigma)
- Octyl-β-D-glucopyranoside (Sigma)
- Trisialoganglioside G_{T_1b} from bovine brain (Calbiochem)
- 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine in chloroform (DOPC; Avanti Polar Lipids) ! CAUTION Chloroform is a carcinogen and can cause liver and kidney damage. It should be handled and disposed of appropriately (see www.osha.gov for further information).
- Lissamine rhodamine B 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (rhodamine-DHPE; Invitrogen/Molecular Probes)
- Anti-cholera toxin beta subunit mouse monoclonal antibody (anti-CTBS; Biodesign Laboratories)
- Botulinum neurotoxin type A from *Clostridium botulinum* (BoNT/A; Metabiologics) ! CAUTION BoNT/A is extremely toxic with a human LD_{50} of ~1 ng/kg. Only trained personnel should work with this toxin, and registration with the Center for Disease Control and Prevention (CDC) may be required (http://www.cdc.gov). Appropriate laboratory safety procedures should be employed. See the following website and references contained therein for additional details (http://pathema.tigr.org/pathema/BoNT_protocols.shtml).
- Affinity-purified polyclonal rabbit IgG antibody against BoNT/A (anti-BoNT/A; Metabiologics)
- Costar flat-bottom EIA and Easy Wash high-binding 96-well microtiter plates (Fisher Scientific)
- PCR primers (DNA Technologies)
- AmpliTaq Gold DNA polymerase (Applied Biosystems)
- Taqman universal PCR mastermix (Applied Biosystems)
- PCR Taqman probe (Applied Biosystems)
- 10× PCR buffer (Invitrogen)
- TOPO TA cloning kit with pCR2.1-TOPO T/A plasmid vector and One-Shot *E. coli* (Invitrogen)
- TRizol Plus RNA purification kit (Invitrogen)
- SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen)
- Hela cells (ATCC)
- Plasmid DNA Mini-Prep kit (QIAGEN)
- TO-PRO-1 DNA intercalating fluorescent dye (Invitrogen/Molecular Probes)
- QIAquick PCR purification kit (QIAGEN)
- 3M sodium acetate (Sigma)
- Glycogen, ultrapure (Sigma)
- Ethanol, absolute (Aldrich)

REAGENTS SETUP

Coating buffer 50 mM carbonate/bicarbonate buffer (Kirkegaard & Perry), pH 9.6

Blocking/dilution buffer 1% (w/v) BSA in PBS, pH 7.8

Wash buffer A 2 mM imidazol/0.02% (w/v) Tween-20 in PBS (Kirkegaard & Perry), pH 7.4

Wash buffer B PBS, pH 7.4

Digestion buffer 10 mM $CaCl_2$/10 mM $MgCl_2$/20 mM HEPES (Sigma), pH 7.8

Lysis buffer 10 mM Triton X-100 in 10 mM borate (Sigma), pH 9.0

EQUIPMENT

- Probe-tip sonicator, Sonic Dismembrator/model 500, with 1/8" probe (Fisher Scientific)
- Dynamic light-scattering spectrometer, Nicomp/model 370 (Particle Sizing Systems)
- Microtiter plate washer, model ELx405 (Bio-Tek)
- ABI PRISM genetic sequencer, model 7700 (Applied Biosystems)
- GeneAmp 9600 for reporter amplification (Perkin-Elmer Corporation)
- Eppendorf model 5417R bench-top centrifuge with model FA 45-24-11 rotor.
- Optima TLX ultracentrifuge and model TLS 55 swinging bucket rotor (Beckman Instruments)

PROCEDURE

Preparation of reporter

1| The double-stranded DNA (reporter) that is encapsulated inside the liposomes serves only as a PCR amplification surrogate for detection and quantification of the corresponding biotoxin target of the assay. Thus, any convenient sequence can be used; however, the sequence should be <100 bp in length to ensure the best amplification efficiency, and to maximize the number of reporters encapsulated into the liposomes. The reporter should also be a sequence not likely to be found in the specimens to be analyzed.

2| We use an 84-base segment derived from the human β_2-microglobin transcript. Since the final assay does not involve a reverse-transcriptase step, this sequence, which spans an intron, will not be found in contaminating human DNA. The following is the complete sequence of the β_2-microglobin reporter used in this assay[10]: 5′-TGA CTT TGT CAC AGC CCA AGA TAG TAA GTG GGA TCG AGA CAT GTA AGC AGC ATC ATG GAG GTT TGA AGA TGC CGC ATT TGG ATT–3′

3| Pellet HeLa cells suspended in PBS buffer, pH 7.4, by centrifugation at 300*g* for 10 min to produce a pellet of ~1 × 10^7 cells. Extract the total RNA from the cell pellet using the TRizol Plus RNA Purification kit by following the manufacturer's instructions.

4| Convert the RNA isolated from the cell pellet to cDNA using the SuperScript First-Strand Synthesis System for RT-PCR (with random primers) by following the manufacturer's instructions.

5| Prepare reporter DNA by first amplifying cDNA from β_2-microglobin transcripts derived from the HeLa cells using β_2M-246F and β_2M-330R primers (*vide infra*). All primer and probe designs were performed using Taqman Probe & Primer Design software (ABI). The primer sequences are:

β_2M—246F(forward) : 5′-TGA CTT TGT CAC AGC CCA AGA TA-3′

β_2M—330R(reverse) : 5′-AAT CCA AAT GCG GCA TCT TC-3′

Check for the presence of reporter DNA by agarose gel electrophoresis.

6| Clone the amplified reporter into a pCR2.1-TOPO T/A plasmid vector and use this vector to transform One-Shot *E. coli* using the TOPO TA cloning kit by following the manufacturer's instructions.

7| Extract the plasmid DNA using the Plasmid DNA Mini-Prep kit. Amplify a 328 bp DNA fragment from the above recombinant plasmid using M13 forward and reverse primers. This is done to ensure that only the β_2-microglobin reporter is amplified in the final PCR step (*vide infra*). The M13 primer sequences are:

Forward : 5′-GTA AAA CGA CGG CCA G-3′

Reverse : 5′-CAG GAA ACA GCT ATG AC-3′

8| Amplify the DNA fragment using a protocol of 29 cycles as follows:

Cycle number	Denature	Anneal	Extend
1–25	95 °C for 60 s	55 °C for 1 min	72 °C for 3 min
26			72 °C for 10 min

Use a PCR reaction mixture consisting of

Plasmid DNA	10 ng
10× PCR Buffer	5 μl
10 mM dNTP mix	1 μl
25 mM $MgCl_2$	5 μl
M13 forward primer (0.1 μg ml^{-1})	1 μl
M13 reverse primer (0.1 μg ml^{-1})	1 μl
Nuclease-free water	35.6 μl
Taq polymerase (1 unit μl^{-1})	0.4 μl

Following PCR, confirm the presence of the 328 bp DNA fragment by agarose gel electrophoresis.

9| Generate the 85-bp reporter by amplifying the 328-bp fragment using the β_2M-246F and β_2M-330R primer set (15 μM each) using the same PCR conditions as above. Following PCR, confirm the presence of the 85-bp DNA fragment by agarose gel electrophoresis.

10| Purify the 85-bp reporter using a QIAquick PCR purification kit and then precipitate the reporter at −20 °C overnight by adding 1/10 (v/v) of 3M sodium acetate, pH 5.2, and three volumes of absolute ethanol containing glycogen (1 ng ml^{-1}) as a carrier.

11| Centrifuge the DNA solution at 16,000*g* for 25 min at 23 °C. Wash the DNA pellet with 70% ethanol and dry it under a stream of nitrogen. Confirm the purity of the reporter by agarose gel electrophoresis.

12| Dissolve the dry reporter in 500 μl of 10 mM Tris, pH 7.4. Determine the reporter concentration by measuring the DNA solution absorbance at a wavelength of 260 nm. An absorbance of 1.0 at 260 nm corresponds to a reporter concentration of 50 μg ml^{-1}. The weight concentration of the reporter can be converted into molar concentration using the reporter molecular weight, which is 54.9 kDa.

13| Finally, dilute the reporter to a concentration of 667 μg ml^{-1} and store at −80 °C.

■ **PAUSE POINT** The reporter is stable for at least 2 years at this temperature.

An alternative to preparing your own reporter is to purchase it commercially. There are numerous vendors (e.g., Integrated DNA Technologies) that offer synthetic PAGE-purified oligonucleotides of any desired sequence and length. A second option is to purchase an optimized set consisting of a reporter, forward and reverse primers and Taqman probe. Applied Biosystems is one commercial source of such optimized reagents. This approach greatly simplifies the LPCR assay, but is more expensive.

Preparation of liposome detection reagents

14| Dissolve 58 mg of DOPC, 5.8 mg of rhodamine-DHPE and either 3 mg of monosialoganglioside G_{M1} or 4.2 mg of trisialoganglioside G_{T1b} in chloroform to a final volume of 2–4 mL. The molar ratio of the three components in this solution is 92.2:5.4:2.4. The rhodamine-DHPE is added to determine the concentration of the liposome solution and as a visualization aid during purification. It does not interfere with the real-time PCR measurement.

15| Add the solution to a test tube, and remove the chloroform by incubation in a water bath (heated to 45 °C) under a stream of nitrogen gas.

! CAUTION Chloroform is a carcinogen and can cause liver and kidney damage. It should be handled and disposed of appropriately (see http://www.osha.gov for further information). Evaporation should be carried out under a chemical fume hood.

16| Remove residual chloroform by incubation in a vacuum dryer for at least 4 h.

17| Disperse the dry lipid mixture in 1 ml of 10 mM Tris buffer, pH 7.4, to yield a total lipid concentration of 80 mM. Use a vortex mixer set on high speed and continue until there is no lipid film remaining on the sides or bottom of the tube.

18| Prepare small unilamellar vesicles (SUVs) by sonication with a probe-tip sonicator. Use a sonication program of 10 cycles of 4 min on/1 min off. Immerse the tube in an ice bath throughout the process to minimize sample heating.

19| Centrifuge the resulting SUVs at 1,500*g* in a microcentrifuge for 5 min to remove undispersed lipid and titanium from the probe tip.

20| Combine SUVs (250 µl, 20 µmol total lipid) and reporter (150 µl, 100 µg).

21| To this mixture, add 600 µl of ethanol/calcium chloride solution (8.3 mM $CaCl_2$ in 16.6 mM Tris, pH 7.4, containing 79% (v/v) ethanol). Add the solution dropwise over approximately 30 s with maximum vortex mixing.

▲ CRITICAL STEP The ethanolic/calcium chloride solution must be added slowly to the rapidly vortexed liposome-DNA solution to prevent high local concentrations of calcium, which would lead to undispersed DNA-lipid aggregates.

22| Dialyze the resulting DNA-containing large unilamellar liposomes against 500 volumes of PBS, pH 7.4, for 24 h at 4 °C with two changes of buffer[11].

■ PAUSE POINT The liposome mixture can be stored at 4 °C for up to 1 week.

Purification of the liposome detection reagents

23| Mix the liposome suspension (0.2 ml) with 0.4 ml of 30% (w/v) Ficoll dissolved in PBS, pH 7.4, to give a final concentration of 20% (w/v) Ficoll in PBS. Transfer the liposome suspension to an ultracentrifuge tube in a swinging bucket rotor.

24| Gently layer a 1.2-ml volume of 10% (w/v) Ficoll in PBS, pH 7.4, on top of the liposome suspension. Cover the Ficoll layers with a 0.4-ml layer of PBS, pH 7.4.

25| Centrifuge the discontinuous gradient for 30 min at 100,000*g* at 23 °C.

26| Collect the liposomes at the interface between the saline and 10% (w/v) Ficoll layers. Unencapsulated reporter remains in the lowest Ficoll layer.

27| Dialyze the purified liposomes at 4 °C against 500 volumes of PBS, pH 7.4 (12 h), followed by 500 volumes of 10 mM Tris, pH 7.8 (12 h).

28| Store the purified liposome detection reagent under nitrogen in a sealed dark vial at 4 °C.

■ PAUSE POINT The liposome detection reagent can be stored for up to 6 months with little loss of encapsulated reporter. For an alternate liposome purification procedure see **Box 1**.

Preparation of blocking liposomes

29| SUVs are used as a blocking reagent in the microtiter plate assay.

BOX 1 | ALTERNATE LIPOSOME PURIFICATION PROCEDURE

An alternate purification procedure is to degrade the unencapsulated reporter with Dnase I and exonuclease III. The DNA-liposomes are then resolved from the free nucleotides by gel permeation chromatography[19].

To the dialyzed liposomes from Step 22, add 2,000 units of pancreatic DNase I, 300 units of exonuclease III and 5 mM $MgCl_2$ to the external aqueous phase.

Incubate the reaction mixture for 3 h at 37 °C, and then stop the reaction by adding 7 mM EDTA.

Remove the nucleotides and enzymes from the DNA-liposomes by elution from a 5 ml Sepharose CL-4B column equilibrated in 10 mM Tris, pH 7.8. Proceed from Step 28.

30| The blocking SUVs are prepared using a lipid mixture of DOPC and rhodamine-DHPE (94.6:5.4), but no ganglioside or reporter is added during the preparation.

31| Prepare and purify the blocking SUVs as described above (Steps 15–19).

32| Store the purified liposome detection reagent under nitrogen in a sealed dark vial at 4 °C.
■ **PAUSE POINT** The blocking liposomes can be stored for up to 3 months.

Characterization of the liposome detection reagents
Determination of total lipid concentration
33| Mix 25–100 µl of liposome solution in a test tube along with 1.5 ml of methanol and 20 µl of 0.1 M NaOH. Vortex the solution and allow it to stand for 5 min.

34| Prepare a blank by substituting PBS for the liposome solution.

35| Read the absorbance of the liposome solution at 560 nm after zeroing the spectrophotometer against the blank.

36| Determine the rhodamine-DHPE concentration using an extinction coefficient of 95,000 $M^{-1}cm^{-1}$ after compensating for the dilution factor.

37| Calculate the total lipid concentration based upon the mole percent of rhodamine-DHPE in the original lipid mixture[8].
▲ **CRITICAL STEP** Normally, the liposome detection reagents require no further characterization beyond the calculation of the total lipid concentration. The effect on the assay of variations in the ganglioside concentration or the number of encapsulated reporters per liposome is compensated for by determining a standard curve using known concentrations of biotoxin. However, a brief discussion of a more complete characterization of the liposome detection reagents is provided for those desiring to develop their own assays using different gangliosides or reporters.

Determination of liposome concentration
38| Determine the hydrodynamic diameter of the liposomes using any dynamic light scattering spectrometer that can measure particle diameters from 10 to 1,000 nm. Follow the manufacturer's instructions for the use of the spectrometer. If a suitable spectrometer is not available, a diameter of 150 nm can be assumed[4].

39| Estimate the number of lipid molecules per liposome (N_{tot}) using equation (1), where d is the hydrodynamic diameter of the liposomes as determined by dynamic light scattering. This equation assumes a bilayer thickness of 4 nm and a lipid headgroup area of 0.71 nm^2 for phosphatidylcholine. The contribution of ganglioside and rhodamine-DHPE to the average headgroup area are ignored in this approximation.

$$N_{tot} = (4.43 nm^{-2}) \times [d^2 + (d - 8nm)^2] \quad (1)$$

40| Estimate the concentration of liposomes in the solution (L_{tot}) using equation (2):

$$L_{tot}(\mu mol\ ml^{-1}) = [total\ lipid(\mu mol\ ml^{-1})]/N_{tot} \quad (2)$$

Determination of reporter concentration
41| Mix 100 µl of liposome solution with 900 µl of 100 mM Octyl-β-D-glucopyranoside. Vortex the mixture and incubate at 37 °C for 15 min. Prepare a corresponding blank using 100 µl of PBS, pH 7.4.

42| Read the optical absorbance of the sample at 260 nm and subtract the corresponding reading for the blank.

43| The total reporter concentration (R_{tot}) is calculated using equation (3):

$$R_{tot}(\mu g\ ml^{-1}) = A_{260} \times 0.020(\mu g\ ml^{-1}) \times 10 \quad (3)$$

where A_{260} is the optical absorbance of the sample, 0.020 μg ml^{-1} is the absorbance of a 1 μg ml^{-1} solution of β_2-microglobin reporter, and 10 is the dilution factor.

Determination of the number of reporters per liposome

44| Add 1 μl of 1 mM TO-PRO-1 in dimethylsulfoxide to a 1 ml solution of DNA-liposomes diluted 100-fold in PBS, pH 7.4. Prepare a scattering blank by substituting 1 μl of dimethylsulfoxide for the TO PRO-1 solution[11].

45| Measure the fluorescence emission of the liposome solution at 531 nm using an excitation wavelength of 514 nm and 5 nm slit widths. Subtract the fluorescence of the blank solution from that of the sample.

46| Add 20 μl of 100 mM Triton X-100 to both liposome solutions, vortex, and allow the solutions to incubate in capped tubes at 37 °C for 15 min. This serves to rupture the liposomes.

47| Re-measure the fluorescence emission of the liposome solution at 531 nm using an excitation wavelength of 514 nm and 5 nm slit widths. Subtract the fluorescence of the blank solution from that of the sample and correct for the dilution of the detergent solution.

48| Determine the percent encapsulation as the ratio of fluorescent intensity before to that after the addition of 20 μl of 100 mM Triton X-100 to rupture the liposome detection reagents.

49| Determine the concentration of encapsulated reporter by using the percent encapsulation and the total reporter concentration determined in Step 43.

50| Determine the number of reporters per liposome by dividing the concentration of encapsulated reporter by the liposome concentration (L_{tot}) determined in Step 40.

LPCR microtiter plate assay for CTBS

51| Coat each well of a 96-well EIA high-binding flat plate with 150 μl of anti-CTBS mouse monoclonal antibody (1.0 μg ml^{-1}) in coating buffer.

52| Cover the microtiter plate with a plate sealer and incubate the plate at 4 °C on a plate shaker at 600 r.p.m. for 18 h.

53| Aspirate the coating buffer and wash the plate wells five times with wash buffer A using a microtiter plate washer.

54| Add 300 μl of blocking buffer to each well and incubate the plate for 2 h at 23 °C.

55| Aspirate the blocking buffer and wash the wells twice with wash buffer A.

56| Add 150 μl of serially diluted CTBS (concentration range: 10^{-14} to 10^{-19} M in dilution buffer) or 150 μl of dilution buffer (blank) to the plate wells. Also, include a 'no template' control. Prepare 3–5 replicates for each antigen concentration, including the blank and control. Incubate the plate at 23 °C for 1 h.

57| Aspirate the sample solutions and wash the wells five times with wash buffer A and twice with wash buffer B.

58| Add 150 μl of blocking liposomes (2.0 μmol ml^{-1} total lipid, diluted 1:1,000 in dilution buffer) to the plate wells, and incubate the plate at 23 °C for 1 h.

59| Aspirate the blocking liposome solution and wash the plate three times with wash buffer B.

60| Add 150 μl of the monosialoganglioside G_{M1}-containing liposome detection reagent (0.8 μmol ml^{-1} total lipid, diluted 1:1,000 in dilution buffer) to the wells, and incubate the microtiter plate at 23 °C for 1 h.

61| Aspirate the detection liposomes and wash the wells ten times with wash buffer B.

62| Degrade any unencapsulated DNA by adding 150 IU of pancreatic DNase I in 100 μl of digestion buffer to each plate well. Cover with a plate sealer, and incubate the plate at 37 °C on a plate shaker with gentle shaking for 30 min (ref. 12).

63| Heat the plate at 80 °C for 10 min to inactivate the DNase I. Aspirate the enzyme solution and wash the wells five times with wash buffer B.

64| Add 100 µl of lysis buffer to the wells, cover with a plate sealer and incubate the plate on a shaker at 600 r.p.m. for 15 min at 23 °C. The lysis buffer serves to rupture the membranes of the liposomes, which releases the encapsulated reporters. Blocking with a nonspecific DNA to prevent loss of reporter is not required.

▲ **CRITICAL STEP** All of the above steps are critical to the success of the LPCR assay. A high concentration of DNase I is used since DNA absorbed to the plate walls or the outer surface of the liposomes can be difficult to digest. It is acceptable to use a partially purified grade of DNase I (non RNase free) to minimize cost.

■ **PAUSE POINT** The plate can remain sealed overnight prior to analysis by real-time PCR.

? TROUBLESHOOTING

LPCR microtiter plate assay for BoNT/A

65| The LPCR microtiter plate assay for BoNT/A is carried out as described above with the following modifications.

- Trisialoganglioside G_{T1b}-containing liposomes are used as the detection reagent due to the high affinity of this ganglioside for BoNT/A[13].
- Corning flat-bottom EasyWash high-binding 96-well plates are used.
- The capture antibody (anti-BoNT/A) concentration in coating buffer is 2.5 µg ml^{-1}.

Real-time PCR

66| Add a 2-µl aliquot from each microtiter plate well to a 50-µl PCR reaction mixture prepared from Taqman universal PCR Mastermix, which contains Taqman buffer A; 3.5 mM $MgCl_2$; 200 µM each of dATP, dCTP and dGTP; 400 µM dUTP; 1.25 units of AmpliTaq Gold; and 0.5 units of AmpErase UNG.

67| Add forward and reverse primers (300 nM each) along with the probe (200 nM).

68| Set up and initiate a PCR protocol consisting of a 2-min UNG incubation step at 50 °C and a 10-min AmpliTaq Gold activation step at 95 °C. Then perform 40 cycles of PCR, where each cycle consists of a 15-s denaturation step at 95 °C and a 1-min annealing/extension step at 60 °C.

69| The forward (β_2M-246F) and reverse (β_2M-330R) primer sequences were given in Step 5. The fluorescent probe is:

5′-[VIC] TGA TGC TGC TTA CAT GTC TCG ATC CCA[TAMRA]-3′

Data analysis

70| For each antigen concentration in the dilution series, including the blank, calculate the mean C_t value and the standard deviation of the 3–5 replicate measurements from the real-time PCR analysis. The no template control should have a C_t value of 37 to 40. A lower value could indicate the presence of reporter contamination in the PCR reaction mixture[14].

71| Construct a standard curve by plotting the average C_t values and their standard deviations for the serially diluted antigen versus the $\log_{10}$ of the antigen concentration. Perform a linear regression analysis of the data to obtain the equation for the standard curve and the 95% confidence limits. The linear correlation coefficient should be ≥ 0.98. Data analysis is performed with Origin version 7.0 or equivalent software.

72| Determine the detection threshold of the LPCR assay, which is defined as the average C_t value of the blank minus three times the standard deviation of the blank, as is used for immuno-PCR assays[5,15].

73| Convert the C_t value of an unknown sample into toxin concentration by interpolation using the standard curve and linear regression equation determined with the serially diluted standards. The unknown sample must be within the dynamic range of the standard curve, which is typically 5–6 orders of magnitude.

▲ **CRITICAL STEP** Always prepare the standard curve using the same sample matrix as the unknowns. For example, if the samples to be analyzed are urine specimens, the standards should also be prepared in urine. Always include serially diluted standards with every plate to ensure the highest possible precision in the LPCR measurements. The PCR amplification efficiency[16] (E) can normally be determined from the slope of the standard curve as $E = 10^{-1/\text{slope}}$. If the efficiency of a PCR reaction is 100%, a $\log_{10}$ increase in reporter concentration will require about 3.3 cycles, which yields a value of 2 for E. For the LPCR assays reported here, the slopes are ~1, thus $E > 2$ indicating efficiencies greater than 100%. This arises from the fact that the slopes of the LPCR assays are a function of the change in biotoxin concentration superimposed upon a relatively constant level of nonspecific binding of the DNA-liposomes. For this reason, the apparent amplification efficiencies are greater than 100%, a fact that does not in itself compromise the accuracy of the assay[16].

● TIMING

Steps 1–13: 2 d (if amplifying existing β_2-microglobin transcript), or 2–4 weeks (if cloning is required)
Steps 14–22: 2 d
Steps 23–28: 1 d
Steps 29–32: 6 h
Steps 33–50: 2 h
Steps 51–65: 4 h
Steps 66–73: 2 h

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

TABLE 1 | Troubleshooting table.

Problem	Possible reasons	Solutions
Assay background high		
Steps 54, 56, 58, 60	Poor quality BSA	Use RIA-grade BSA
Steps 29–32, 58	Poor SUV blocking	Use fresh blocking liposomes or a higher SUV concentration
Step 60	Detection reagent concentration too high	Use a higher dilution of DNA-liposomes
Steps 62–63	Incomplete DNA digestion	Use fresh DNase I, a higher enzyme concentration, or a longer digestion time
Steps 51–64	Improper reagents or assay conditions	Check buffer compositions, check proper operation of plate washer, increase number of wash cycles
Steps 66–69	Contaminated PCR reaction mixture	Check no-template control, if C_t below 37 replace PCR reagents
Loss of assay sensitivity		
Steps 14–28	Detection reagent too old	Prepare fresh DNA-liposomes
Step 60	Detection reagent concentration too low	Use a lower dilution of DNA-liposomes
Step 60	Non-optimal pH	Use pH 7.8 for detection reagent binding
Step 51	Capture antibody too old or too dilute	Use fresh capture antibody or decrease capture antibody dilution
Steps 14–28	Too little ganglioside in detection reagent	Prepare fresh DNA-liposomes using proper lipid composition
Poor dynamic range		
Step 60	Formation of confluent liposome monolayer[8]	Use a lower concentration of detection reagent
Steps 66–69	Poor PCR reaction conditions	Check real-time PCR instrument and protocol. Replace PCR reagents
Poor reproducibility		
Step 54	Too few replicate measurements	Increase the number of replicates per sample. Particularly important for low toxin concentrations[4]
Steps 49–67	Poor technique	Check for proper operation of pipetters, plate washer, etc.

ANTICIPATED RESULTS

For biotoxin assays performed in deionized water, detection thresholds down to 10–50 molecules in 150 µl of water are typical (zeptomolar (10^{-21}M) to attomolar (10^{-18}M) concentration range). The lower concentration limit is determined predominately by the binding affinities of the capture antibody and ganglioside for the biotoxin. The affinity of gangliosides for biotoxins can vary widely[1,9]. For example, the detection threshold for an assay for tetanus toxin in deionized water using G_{T1b}-containing liposomes is ~325 molecules (unpublished data) due to the lower binding affinity of G_{T1b} for tetanus toxin relative to BoNT/A.

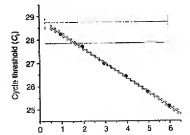

Figure 2 | Results of an LPCR assay of human urine spiked with CTBS. A urine specimen was collected from a healthy human male volunteer. The urine specimen was spiked with cholera toxin beta subunit (CTBS), filtered through a 0.2-µm polycarbonate filter to remove any particulates, and the pH of the specimen was adjusted to 7.8 using 0.1 M NaOH. Four replicate liposome polymerase chain reaction (LPCR) measurements were carried out for each of six serial dilutions of CTBS in the urine specimen (concentration range: 10^{-14} to 10^{-19} M) plus an unspiked urine blank. A plot of the average serial dilution cycle threshold (C_t) values versus the log of the number of CTBS molecules per plate well for the four replicate measurements is shown. The solid black circles are the average serial dilution C_t values. The solid red line is a linear regression fit to the C_t values, and the dashed blue lines are the 95% confidence limits. The solid horizontal orange line denotes the average blank C_t value. The standard deviation of the blank is drawn at each end of this line. The solid horizontal green line that intersects the linear regression line indicates the detection threshold of the assay. The detection threshold for this LPCR assay is 43 ± 10 molecules of CTBS (0.5 attomolar or 0.09 fg ml^{-1}). The assay dynamic range is almost five orders of magnitude. The slope of the linear regression fit of the data is -1.02 ($r^2 = 0.998$).

The selection of the capture antibody is critical, as it cannot compete with the ganglioside for the same epitope. For example, attempts to create and LPCR assay for ricin using the monoclonal antibody 2R1 (clone CP23)[17] were unsuccessful (unpublished data) as both apparently compete for the same epitope on the ricin A-chain. In general, polyclonal antibodies are more effective than monoclonal antibodies for use as the capture antibody in the LPCR assays. Biotoxin assays using environmental or biological specimens have higher detection thresholds due to the higher background (DNA-liposome non-specific binding) resulting from the more complex matrix. Detection thresholds of 50–500 molecules in 150 μl of solution [attomolar (10^{-18}M) concentration range] are typical for these more complex specimens.

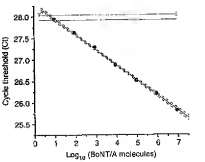

Figure 3 | Results of an LPCR assay of deionized water spiked with BoNT/A. Deionized water (18 MΩ) was spiked with botulinum neurotoxin type A (BoNT/A), filtered through a 0.2-μm polycarbonate filter to remove any particulates, and the pH of the specimen was adjusted to 7.8 using 0.1 M NaOH. Four replicate LPCR measurements were carried out for each of six serial dilutions of BoNT/A (concentration range: 10^{-16} to 10^{-19} M) plus an un-spiked water blank. A plot of the average serial dilution cycle threshold (C_t) values versus the log of the number of BoNT/A molecules per plate well for the four replicate measurements is shown. The symbols are as defined in **Figure 2**. The detection threshold is 12 ± 4 molecules of BoNT/A (0.1 attomolar or 0.02 fg ml^{-1}). The assay is linear over approximately five orders of magnitude. The slope of the linear regression fit of the data is −0.632 (r^2 = 0.998).

Representative results obtained for LPCR assays of CTBS and BoNT/A are the following[4] **(Figs. 2 and 3)**:

- CTBS in deionized water. The detection threshold is 10 ± 3 molecules of CTBS [17 yoctomoles (17 × 10^{-24}) derived from a 113-zeptomolar solution (0.02 fg ml^{-1})] based upon the linear regression and 95% confidence limits derived from the sample data. The dynamic range of the assay is almost five orders of magnitude.
- CTBS in human urine. The detection threshold is 43 ± 10 molecules of CTBS (71 yoctomoles derived from a 0.5-attomolar solution (0.09 fg ml^{-1})). The dynamic range of the assay is almost six orders of magnitude.
- CTBS in farm runoff water. The detection threshold is 377 ± 168 molecules of CTBS [0.6 zeptomoles derived from a 4-attomolar solution (0.75 fg ml^{-1})]. The dynamic range of the assay is almost five orders of magnitude.
- BoNT/A in deionized water. The detection threshold is 12 ± 4 molecules [20 yoctomoles derived from a 0.1 attomolar solution (0.02 fg ml^{-1})]. The assay is linear over approximately five orders of magnitude.

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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